A novel missense mutation in the Sodium Bicarbonate Cotransporter (NBCe1/SLC4A4) causes proximal tubular acidosis and glaucoma through ion transport defects

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Running title: S427L-NBCe1 is an ion transport defect

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SUMMARY

In humans and terrestrial vertebrates, the kidney controls systemic pH in part by absorbing filtered bicarbonate in the proximal tubule via an electrogenic Na⁺/HCO₃⁻ cotransporter (NBCe1/SLC4A4). Recently, human genetics revealed that NBCe1 is the major renal contributor to this process. Igarashi et al. showed that homozygous point mutations in NBCe1 cause proximal renal tubular acidosis (pRTA), glaucoma and cataracts.

We have identified and functionally characterized a novel, homozygous, missense mutation (S427L) in NBCe1, also resulting in pRTA and similar eye defects without metal retardation. To understand the pathophysiology of the syndrome, we expressed wild-type (wt) and S427L-NBCe1 in *Xenopus* oocytes. Function was evaluated by measuring intracellular pH (HCO₃⁻ transport) and membrane currents using microelectrodes.

HCO₃⁻ elicited currents for S427L were ~10% of wt-NBCe1, and CO₂-induced acidification was ~4-fold faster. Na⁺ dependent HCO₃⁻ transport (currents and acidification) was also ~10% of wt. Current-voltage (I-V) analysis reveals that S427L has no reversal potential in HCO₃⁻, indicating that under physiological ion gradient conditions NaHCO₃ could not move out of cells as is needed for renal HCO₃⁻ absorption and ocular pressure homeostasis. I-V analysis without Na⁺ further shows that the S427L-mediated NaHCO₃ efflux mode is depressed or absent. These experiments reveal that voltage and Na⁺ dependent transport by S427L-hkNBCe1 is unfavorably altered, thereby causing both insufficient HCO₃⁻ absorption by the kidney (proximal RTA) and inappropriate anterior chamber fluid transport (glaucoma).
INTRODUCTION

Maintenance of body fluid pH within a narrow range is critical for a wide variety of essential biochemical and metabolic functions. The kidney plays a key role in normal homeostasis, due to its ability to reclamation HCO$_3^-$ and excrete acid. Renal tubular acidosis (RTA) is a clinical syndrome characterized by hyperchloremic metabolic acidosis resulting from two physiologically distinct disorders of renal acidification. In distal RTA (dRTA) the kidney fails to produce an appropriately acid urine in the presence of systemic metabolic acidosis or after acid loading, due to impaired hydrogen ion secretion in the distal nephron. Both autosomal dominant (OMIM #179800) (1-5) and autosomal recessive (OMIM #602722) (6,7) patterns have been observed in kindreds with primary dRTA, and the spectrum of clinical severity is wide. Proximal RTA (pRTA) is caused by an impairment of bicarbonate absorption in the proximal tubule (PT), and is characterized by a decreased renal HCO$_3^-$ threshold (8).

The kidney receives 20% of the cardiac output, translating to a renal blood flow (RBF) of 1 L/min in humans, i.e., 1,800 L/day. Daily glomerular filtration is ~10% of RBF (~180 L/day of ultrafiltrate). The PT isotonically absorbs ~67% of filtered ions, solutes and water, i.e., ~120 L/day of isotonic solution. Thus, the PT is a major player in ion and H$_2$O (volume) homeostasis. The PT can increase HCO$_3^-$ absorption by up to 90% of total via “new HCO$_3^-$ synthesis,” i.e., ammoniagenesis. This HCO$_3^-$ absorption is a transepithelial process. First, HCO$_3^-$ crosses the apical membrane as CO$_2$ after being titrated with H$^+$ (from the NHE3 Na$^+$-H$^+$ exchanger). This process is facilitated by an apical carbonic anhydrase IV (CAIV). Once CO$_2$ and H$_2$O are in the proximal tubule cells, carbonic anhydrase II (CAII) facilitates the reformation of H$^+$ and HCO$_3^-$.

Finally, HCO$_3^-$ exits the basolateral membrane, into the blood, coupled to Na$^+$ via the electrogenic Na$^+$/HCO$_3^-$ cotransporter (NBCe1)(9). Thus, when HCO$_3^-$ is absorbed, Na$^+$ and water come along to maintain isotonic absorption. In humans, this proximal absorption is ~340 gm (0.75 lb) of NaHCO$_3$/day.

A rare syndrome characterized by profound pure pRTA (blood pH <7.1 and blood [HCO$_3^-$] = 5-11 mM), short stature, mental retardation and bilateral glaucoma with or without...
cataracts and band keratopathy has been described so far in only 3 families (10,11). That is, Igarashi et al. identified three homozygous SLC4A4 mutations (R298S; R501H; Q29X) in three unrelated Japanese patients (10,11). These mutations reveal that NBCe1 the major HCO$_3^-$ transporter of the proximal tubule and a major kidney controller of systemic acid-base status.

We have identified a novel, SLC4A4 missense mutation, S427L, located in the beginning of the predicted first transmembrane span (TM1) (9,12-14) in a female Israeli patient with uncompensated pRTA. Here we provide a patient description, biophysical characterization of the S427L-hkNBCe1 transport defect and additional insight to the pathology of this genetic disorder.

 Portions of this work have been reported in preliminary form (15-17).

**EXPERIMENTAL PROCEDURES**

*Patient description*

The patient is a 44-year-old Jewish-Georgian woman with pRTA, short stature (140 cm), normal intelligence, deformed teeth and blindness (Fig 1a). Bilateral glaucoma was diagnosed in early childhood. She underwent several eye operations for high intra-ocular pressure and was treated with acetazolamide from 6 yrs. Bilateral cataracts and corneal opacity developed over the years. She was completely blind at 16. Metabolic acidosis was first noted when she was admitted to the hospital for rectal bleeding at 33 yrs. Stopping acetazolamide treatment did not improve her systemic acid-base status and thus was not the cause of the renal or ocular pathologies.

The patient is the daughter of non-consanguineous parents. Her father died of end stage renal failure. Her mother suffers from mixed connective tissue disease but has no obvious renal or vision abnormalities. A brother and a sister are healthy. No one else in the family has pRTA, eye pathologies, short stature or poor dentition (Fig 1a).

At diagnosis, serum analysis revealed the following levels: Na$^+$ 137 mEq/L, K$^+$ 3.5 mEq/L, Cl$^-$ 122 mEq/L, creatinine 0.7 mg%, urea 30 mg%. Liver function tests and serum amylase were normal. Blood gas analysis revealed a pH of 7.09, [HCO$_3^-$] of 11 mEq/L and P$_{CO_2}$ of 38 mmHg.
Urine pH was 4.92. Intravenous bicarbonate loading (130 mEq/3 hours) raised blood HCO$_3^-$ level to 16 mEq/L and increased the fractional excretion of HCO$_3^-$ from 0 to 41%, confirming the pRTA diagnosis. Subsequent urinalysis showed a pH of 5.0, osmolality 500 mosm/kg, no glucose, blood or protein. The urinary excretion of amino acids, calcium and phosphate was normal. An abdominal ultrasound revealed normal sized kidneys without nephrocalcinosis. Brain computed tomography showed no calcifications, and skeletal X-rays excluded osteopetrosis.

Oral sodium-bicarbonate and potassium were discontinued after 5 years due to hypertension and edema development. The patient is now taking only CaCO$_3$. Amylase levels, not separated for pancreatic and salivary, have been repeated since diagnosis but remain normal. Recent blood work shows pH 7.215, P$_{CO_2}$ 37 mmHg, and HCO$_3^-$15 mEq/L, still indicating a severe, uncompensated pRTA and / or a mixed metabolic-respiratory acidosis.

The patient, her mother and her sister gave informed consent to participate in this study.

**Sequence analysis of carbonic-anhydrase II (CAII) & carbonic-anhydrase IV (CAIV) genes**

Genomic DNA was extracted from the patient’s peripheral blood leukocytes (PBL). From proximal tubule HCO$_3^-$ absorption models, NHE3, CAII, CAIV, and NBCe1 are several of the proteins involved in transepithelial HCO$_3^-$ absorption. As a start, the coding areas of CAII and CAIV were amplified from genomic DNA by PCR. We used previously described primers for CAII (5) and designed intronic primers for CAIV based on its previously reported genomic structure (18). All PCR products were sequenced by directly (ABI Prism 3100, Applied Biosystem).

**Sequence analysis of NBCe1 cDNA**

At the time of the initial genetic study, the genomic organization of NBCe1 was unknown. Therefore, we performed sequence analysis with the NBCe1 cDNA. PBLs were isolated with Histopaque-1077 (Sigma Chemical, MO USA). RNA was extracted from PBLs
with Tri Reagent (MRC Inc., Ohio USA). First strand cDNA of pNBC1 (SLC4A4-B) used an eAMV reverse transcriptase (-RT) enzyme (Sigma) with a gene-specific reverse primer (GSP, AGAGAGCGCTGTATTATTTGGCCTGTGACC), random nonamers and anchored oligo(dT)23. Nested PCRs were performed using forward (F) and reverse (R) primers:

(i) External (E): \( F1 = 5'\)-ATTACTATAGGATGGAGGATG, \( R1 = 5'\) - TCTGAACATTCTCTCCACCTCTGAT
Internal (In):
\( F2 = 5'\) - GCAGCAGCATCCTAAAAACCTCTCA, \( R2 = 5'\) - CATGGAACACCTCATCAGACATCA

(ii) E: \( F3 = 5'\) - CAGTCTGAATGACATTTCTGATAAACGGGA, \( R3 = 5'\) - AGAGAGCGCTGTATTATTTGGCCTGTGACC
In: \( F4 = 5'\) - ATGATCAAGCTTGCAGATTACTACCCCATC, \( R4 = 5'\) - GATCCAAAGGCGGCGACAAACACCTG
\( F5 = 5'\) - GCAGTTCATGGATCGTCTGA, \( R5 = 5'\) - GCAGGTTACAATGTAGTTTCTGTGACC
\( F6 = 5'\) - CGTGATGCAGAAGCTTCCAACG, \( R6 = 5'\) - GTTGGCAAATACTCTGGGGCCA

The NBCe1 cDNA obtained from PBLs was the pancreatic isoform (SLC4A4-B). Therefore, the kidney-specific 5’end of kNBC1 (NBCe1-A/SLC4A4-A) gene was amplified from genomic DNA of the patient using the following primers:

\( F7 = 5'\) - CGTTCAGAACCAAAGGATAAGAGGGAACAAACGGAATAGAAGAGGGC, \( R7 = 5'\) - CTGCAGAAGTGAAAAATACTGTG.

**Confirmation of the mutation by genomic DNA sequencing**

Genomic DNA was extracted from PBLs of the patient, her mother and her sister. A healthy brother was unavailable for genetic analysis. A 131 bp segment of the SLC4A4 gene comprising the mutation at position 1429 was amplified using the primers F8 / R8 and sequenced (**Fig 1b**):

\( F8 = 5'\) - ACATAAAGAGGAAAGCGCCATT, \( R8 = 5'\) - CCCCAAGCAGTCCTCCTCAAAAGT.
Detection of S427L mutation by restriction enzyme analysis

We used restriction enzyme analysis to reconfirm the presence of mutation in the patient and her family, and to screen 90 healthy control Israelis. As the S427L mutation does not change a restriction site in NBCe1 gene, we introduced by PCR a second mutation (G1431→A), which created a TaqαI restriction site in PCR products of wild type DNA, but not of mutant DNA (C1429→T). Mutagenesis was performed by PCR amplification of genomic DNA, using F8 & R9= 5’-GCCAGATAAATGAAGAGAATTGTC. PCR products (from DNA of the patient, her family and 90 control Israelis) were cut with TaqαI, run on 3% agarose gel and stained with ethidium bromide (Fig 1c).

Analysis of polymorphic markers on chromosome 4q21

To exclude loss of heterozygosity of the SLC4A4 gene in our patient due to a large chromosomal deletion or whole chromosomal loss, we analyzed polymorphic markers located on chromosome 4q21 near SLC4A4 gene. We used fluorescent primers to amplify the following markers from genomic DNA of the patient, her mother and sister: D4S398, D4S392, D4S2964, D4S1534. Results are illustrated in Fig 1d.

Oocyte experiments

hkNBCe1 and S427L cDNAs. The hkNBCe1 cDNA was generated by RT-PCR using oligo-dT primed human kidney poly(A)+ RNA (Clontech, Palo Alto, CA) and primers corresponding to the start and stop of the known sequence (19):

F10 = 5’-ATgTCCACTgAAAATgTggAAgggAAgCCC, R10 = 5’-TTATCAgCATgATgTggCgTTC.

This PCR product was subcloned into TOPO-pCRII (Invitrogen) and sequenced (WM Keck, New Haven, CT). hkNBCe1 was excised from pCRII with BgIII and EcoRI and cloned into the EcoRI site of pTLN, a Xenopus expression vector (13,20). Sense orientation was determined by restriction analysis and verified by sequencing.
The S427L mutation (C→T) was made by site directed mutagenesis using QuikChange (Stratagene, La Jolla, CA) with mutagenesis primers: S427L_F = 5’-caagctctttTggcaattctcttcatctgccc and S427L_R = 5’-gccagataaatgagaaatgtgccAaaaagagcttg.
The single nucleotide change was confirmed by sequencing the entire S427L cDNA.

**Oocyte isolation and injection**

Female *X.laevis* were purchased from Xenopus Express (Beverly Hills, FL). Oocytes were removed and collagenase dissociated as previously described (13). Capped cRNA was synthesized using a linearized cDNA template and the SP6 mMessage mMACHINE kit (Ambion, Austin, TX). Oocytes were injected with 50 nL of hkNBCe1 cRNA (0.2 µg/µL), S427L-hkNBCe1 cRNA (0.5 µg/µL) or water, and incubated at 18°C in OR3 media (13). Since S427L could cause less efficient protein processing than wt, we injected 0.2, 0.5, and 1.0 µg/µL of cRNA. We chose 0.5 to maximize S427L protein expression. Oocytes were studied 3-10 days after injection.

**Electrophysiology**

Experimental solutions were previously described (21). All solutions were either ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.5) or iso-osmotic ion replacements as described (21).

**Two electrodes voltage clamp.** Oocyte currents were recorded with an OC-720C voltage clamp (Warner Instruments, Hamden, CT), filtered at 2-5 kHz, digitized at 10 kHz and recorded using the Pulse software, and data analyzed using the PulseFit program (HEKA, Germany) as previously (21). For periods when I-V protocols were not being run, oocytes were clamped at a holding potential (V_h) of -60 mV; and current was constantly monitored and recorded at 1Hz. I-V protocols consisted of 100 ms steps from V_h to -160 mV and +60 mV in 20 mV steps as previously (21).

**Ion selective microelectrode.** Ion selective microelectrodes were used to monitor intracellular pH (pH_i) of hkNBCe1 and water injected oocytes as previously described (13) using the H+...
ionophore I-cocktail B ion selective resin (Fluka Chemical). Intracellular pH was measured as the difference between the pH electrode and a KCl voltage electrode impaled into the oocyte; and membrane potential ($V_m$) was the potential difference between the KCl microelectrode and an extracellular calomel (9,13) or output $V_h$ for clamped experiments (22,23). pH electrodes were calibrated using pH 6.0 and 8.0 (Fisher Scientific, Pittsburgh, PA) followed by point calibration in ND96 (pH 7.50) as previously (9,13) and had slopes of at least -54 mV/pH unit.

**Calculations.** Oocytes are perfused with ND96 for 5 min at which time Initial pH$_i$ is measured. The solution is switched to CO$_2$/HCO$_3^-$ for 8-10 min (i.e. pH$_i$ and $V_m$ or I plateau) and the Final pH$_i$ is measured. \([\text{HCO}_3^-]_i\) is calculated from the Henderson-Hasselbach equation and buffering power ($\beta_T$) calculated as $\beta_T=\Delta[\text{HCO}_3^-]/\Delta\text{pH}_i$ (24). $\beta_T$ is an apparent $\beta$ in the case of NBCe1-oocytes because the acid-base transporter hkNBCe1 has been functionally “added” to the “true” buffering power of the H$_2$O injected oocytes. Thus, $\Delta\beta_{hkNBCe1}$ can be represented as the difference of $\beta_T$ (hkNBCe1-oocytes) and $\beta_T$ (H$_2$O-oocytes). “ND96” values refer to responses elicited by removal of CO$_2$/HCO$_3^-$. “n” indicates the total # of experiments. Oocytes came from at least two separate donor animals.

**Immunohistochemistry.** Oocytes were washed with PBS, fixed with 4% para-formaldehyde in PBS for 1 h at RT, and processed for cryosectioning as previously described (25). Immunostaining was performed using 1:200 dilution of the primary kNBCe1 antibody (20) and a Cy3 secondary antibody. Epifluorescent images were captured using a Zeiss AxioVert 25 microscope and acquired with an AxioCam digital camera and AxioVision software (Carl Zeiss, Germany) (25).

**RESULTS**

**Genomic screening**

HCO$_3^-$ absorption by the PT is a transepithelial transport process, relying on apical and basolateral transporters and cellular metabolism. Since pRTA could arise from defects in one of
several proximal tubule proteins, we examined genes directly associated with HCO$_3^-$ absorption. No mutations were found in the entire coding areas of CAII or CAIV in the affected patient (not shown).

Sequencing of the patient’s NBCe1 cDNA identified a homozygous missense mutation C1429→T, which changes Ser 427 to Leu (S427L). This mutation is in both patient alleles (Fig 1b). Genomic DNA sequencing (Fig 1b) and restriction enzyme analysis (Fig 1c) confirmed the presence of homozygous mutation in the patient and showed that the patient’s mother and sister are heterozygous carriers. The C1429→T mutation was not detected in 90 unrelated Israelis (not shown).

Analysis of polymorphic markers located on chromosome 4q21 near SLC4A4 gene showed that the patient has two alleles in this area (Fig 1d), and is homozygous only for D4S392, a marker located only 1.5cM from SLC4A4 gene. Haplotype analysis also showed that the sister inherited a different paternal allele than the patient, consistent with her being heterozygous carrier of the NBCe1 mutation.

**Expression of wt-hkNBCe1 in oocytes**

To gain insight to the pathophysiological consequences of the S427L mutation, we cloned the hkNBCe1 cDNA (wt) from human kidney and expressed it in *Xenopus* oocytes (20). Fig 2 shows representative experiments with a control (left) oocyte and a wt-hkNBCe1-injected oocyte (middle). As with *Ambystoma* and rat kidney NBCe1 (9,13), Fig 2 (middle, a’) illustrates that addition of 5% CO$_2$/33 mM HCO$_3^-$ elicits a hyperpolarization (-67 mV, Na$^+$ and nHCO$_3^-$ influx) wt-oocytes. This CO$_2$ causes an acidification (-0.55 pH units @-265x10$^{-5}$ pH units/s; Fig 2, middle, a→b). Na$^+$ removal in CO$_2$/HCO$_3^-$ results in an additional acidification (-18x10$^{-5}$ pH units/s; Fig 2, middle, b→c) and depolarization (+81 mV, b’→c’). A water control has a similar CO$_2$-evoked acidification (-0.46 pH units @-207x10$^{-5}$ pH units/s, Fig 2, left, a→b$’$) and slow depolarization (+13 mV; a’). Na$^+$ removal in this control cell slightly acidified (-11x10$^{-5}$ pH
units/s; left, b→c) and hyperpolarized the cell (-2 mV; b’). Average responses for non-clamped cells are indicated in Table 1.

Further analysis reveals that [HCO$_3$]$^-$ for hkNBCe1 is elevated with respect to the control (10.0 mM vs. 7.4 mM). Total buffering rose from 16.0 mM/pH unit (control) to 18.2 (hkNBCe1). Buffering due to CO$_2$ is increased from 17.0 mM/pH unit (control) to 23.1 (hkNBCe1).

**S427L-hkNBCe1 in oocytes**

S427L-hkNBCe1 shows small voltage responses after CO$_2$/HCO$_3$- (-15.8 mV, Fig 2 right, a’) yet clearly different from wt (Fig 2, left, a’). For this S427L oocyte, CO$_2$/HCO$_3$- caused an acidification (-0.49 pH units) at an initial rate of -269x10$^{-5}$ pH units/s (Fig 2 right, initial a→b). Na$^+$ removal decreases pH$_i$ by -17x10$^{-5}$ pH units/s (Fig 2 right, b→c) and causes a depolarization (+22.7 mV, b’). Cellular [HCO$_3$]$^-$ was 7.4 mM, equivalent to the water control. $\beta_T$ was 14.9 mM/pH unit and $\beta_{CO2}$ was 16.8 mM/pH unit, both similar to control values. Averages from several experiments show that the unclamped buffering of S427L is not statistically different from water-injected controls (Table 1).

Decreased S427L function was not due to decreased membrane protein expression. We cryosectioned and immunostained the oocytes with a kNBC1 antibody (Fig 2 bottom)(20). Fig 2 illustrates that control, water-injected oocytes do not make NBCe1 immunoreactive protein (left) and that the membrane protein associated with wt (middle) or S427L expression is similar (right).

**Control of membrane potential ($V_m$) effects wt- and S427L-hkNBCe1 function**

Voltage clamp experiments indicated that the S427L transport defect was more severe than initial unclamped pH$_i$ experiments indicated. To recreate the PT basolateral membrane environment, we voltage clamped our oocytes to -60 mV while simultaneously measuring pH$_i$ as
previously for other transporters (22,23). The S427L phenotype as well as the differences between control oocytes and S427L-hkNBCe1 were more obvious in these experiments (Fig 3, Table 2) due to greater experimental control.

CO$_2$/HCO$_3^-$ addition changes both the extent and rate of acidification. A water control oocyte acidifies by -0.63 pH units at -472 x10$^{-5}$ pH units/s (Fig 3, left, a→b). A S427L oocyte acidifies by -0.38 pH units at -330x10$^{-5}$ pH units/s (Fig 3, right). Table 2 shows that under voltage clamp conditions CO$_2$ induced acidification for S427L and control oocytes is similar. By comparison, a wild-type hkNBCe1 injected oocyte only acidifies by -0.11 pH units at -99x10$^{-5}$ pH units/s (Fig 3, middle, a→b). That is, clamped hkNBCe1 oocytes transport enough HCO$_3^-$ (18.1 mM) into the oocyte to buffer the normal CO$_2$-induced acidification by ~ 60%.

Currents measured by these solution changes are quite sensitive. Positive current indicates influx of Na$^+$ + nHCO$_3^-$ (net “-”) into the cell, while negative current indicates efflux. Addition of 33 mM HCO$_3^-$ to wt-hkNBCe1 elicits a +1100 nA peak current (Fig 3, middle, A). The same addition to S427L causes a +99 nA current (Fig 3, right) compared to -27 nA in control oocytes (Fig 3, left).

By controlling $V_m$ (clamped to -60 mV), removal of Na$^+$ (resulting in Na$^+$ + nHCO$_3^-$ efflux as in the PT) reveals obvious acidification rate and current differences between control (+35x10$^{-5}$ pH units/s; -14 nA), hkNBCe1 (-253x10$^{-5}$ pH units/s; -1103 nA) and S427L (-26x10$^{-5}$ pH units/s; -163 nA) (Fig 3, c→d). The pooled data in Table 2 reiterate these results. These $d$PH$_i$/dt data resulting from Na$^+$ removal in Table 1 and Table 2 are initial rates. In the case of hkNBCe1, this acidification rate is nearly constant for 3 minutes thereby decreasing pH$_i$ by 0.3 pH units and would continue to less than 7.0 if the absence of Na$^+$ persisted. However, for S427L the initial rate is not maintained and pH$_i$ does not significantly change from ~7.0 observed in control oocytes. Specifically, Table 2 enumerates $\Delta$PH$_i$ (0Na$^+$ CO$_2$), .i.e., NaHCO$_3$ efflux magnitude, after 3 min for wt (-0.198 ± 0.031 pH units) and S427L (-0.001 ± 0.007 pH units). The ratio of these NaHCO$_3$ effluxes is 0.3% rather than 10% as calculated from the initial rates ($d$PH$_i$/dt). The $V_m$ clamped data illustrate that the unclamped experiments underestimate the magnitude of the
S427L defect. Furthermore, the ΔpHi (0Na+ CO2) data illustrate that S427L does not work well in the NaHCO3 efflux mode.

Total buffering for an unclamped hkNBCe1 oocytes is 32.1±5.6 mM/pH unit (13.9±2.0 mM [HCO3−]). Clamping hkNBCe1 oocytes raises βT to 127.8 mM/pH unit by raising [HCO3−]i to 16.1±1.0 mM. Nevertheless, for controls, clamping does not significantly change [HCO3−]i (9.4±1.8 mM clamped vs. 8.0±0.3 unclamped) or βT (22.9±4.6 mM/pH unit clamped vs. 18.0±1.0 unclamped). For S427L clamping also does not statistically affect [HCO3−]i (7.6±0.5 mM clamped vs. 7.6±0.6 unclamped) or βT (18.5±1.7 mM/pH unit clamped vs. 15.7±1.9 unclamped).

Voltage dependence of wt- and S427L-NBCe1

To further examine the S427L phenotype, we performed rapid voltage steps and monitored the current responses using the same solution protocol as in Fig 3 (at points A-D) and previously (21). Fig 4 shows the results of these experiments: HCO3− addition (initial=HCO3A and steady-state =HCO3B), Na+ removal (0Na-HCO3−) and removal of CO2/HCO3− (ND96-w). HCO3− elicited currents were subtracted from the currents in ND96 just prior to CO2/HCO3− addition. The instantaneous HCO3− current (HCO3A) reveals an apparent reversal potential (Erev) of -120 mV, while at steady-state (HCO3B, ~5 min when pHi has stabilized) reveals (Erev)hkNBCe1 = -83.0±4.8 mV (Fig 4, middle). Erev is the voltage at which the steady-state current changes directions, i.e., positive current (NaHCO3 influx) to negative current (NaHCO3 efflux). HCO3B was reported in our analysis of rat NBCe1 (21) and the hkNBCe1 current-response is similar. No HCO3− elicited currents are observed in control oocytes (Fig 4, left). The S427L response to this voltage clamp protocol shows obvious but attenuated NBCe1 activity (Fig 4, right). In the steady-state S427L does not have an Erev nor shows negative currents. Mere current reversal (for example, +200 nA to +100 nA) means that NaHCO3 influx has decreased rather than true NaHCO3 efflux. Negative current and continued acidification in the absence of Na+ would indicate true reversal of S427L mediated NaHCO3 transport (NaHCO3 efflux). That is, it is not possible to use Vm (the electrical driving force) to move Na+ + HCO3− out of the S427L oocyte. In other words, S427L does not appear to efficiently work in the direction needed for proper PT and ocular anterior chamber function.
This S427L transport defect is further highlighted by Na⁺ removal. Na⁺ replacement with choline in hkBce1 supports outward currents at +Vₘs (influx not efflux). S427L oocytes respond yet differently. First IS427L at -60 mV with “0 Na⁺” is ~ 10% of the wt response. Second, the S427L I-V response has a different shape than wt. Specifically, though S427L oocytes have slight inward currents (current reversal) with respect to HCO₃⁻ elicited currents, however, the currents are not less than 0. Negative current (I < 0) is defined as NaHCO₃ efflux, since a current reversal, that is not less than 0, could result from decreased NaHCO₃ influx rather than actual NaHCO₃ efflux. In fact, -160mV does not electrically force S427L to have net NaHCO₃ efflux (Fig 4, HCO₃B). Removal of CO₂/HCO₃⁻ from solutions (Fig 3, D; Fig 4, ND96-w) shows that S427L oocytes have difficulty with NaHCO₃ efflux driven by 0HCO₃⁻ (-81±56 nA @ +60 mV vs. -451±208 nA for wt).

Fig 5 further illustrates the wt and S427L I(HCO₃) (Fig 5a) and the difference currents for Na⁺ removal in continued HCO₃⁻ (Fig 5b; Fig 3 “C-B”). For an easier qualitative comparison, the IS427L is amplified by 10-fold. As indicated wt has an Erev while at -160 mV S427L does not support NaHCO₃ exit from oocytes (I < 0) and IS427L appears to rectify. Plotting the current reversals associated from 0NaHCO₃ (Fig 5b) shows that ISknBce1 strongly rectifies but is always inward (NaHCO₃ efflux). S427L does not rectify and shows an Erev of about -150 mV.

**DISCUSSION**

We report a new missense mutation in NBCe1 (SLC4A4) from an Israeli kindred. As reported for other NBCe1 mutations (11,26), S427L is a homozygous, single nucleotide transition (C1429→T). The mother and sister, who are heterozygous carriers of the mutation are phenotypically normal. The patient’s father died, so we could not show that he carried the mutation, too. However, as both parents belong to a small close community of Georgian Jews, it is very likely that they had a common ancestor and hence carried the same mutation. Our data cannot exclude a loss of heterozygosity of the SLC4A4 gene, but a whole chromosomal loss or a large chromosomal deletion, were excluded by marker analysis (Fig 1d). In addition, previous reports of a very similar phenotype in Japanese patients with NBCe1 point mutations (11,26),
support our belief that the clinical features of our patient are the result of the novel S427L-NBCe1 mutation.

Though the patient has a persistent pRTA, HCO$_3^-$ therapy helps (latest blood gases: pH 7.215, P$_{\text{CO}_2}$ 37 mmHg, [HCO$_3^-$]=15 mM). The serious clinical pathophysiology is dominated by the kidney and eye: pRTA, bilateral glaucoma and cataracts. Thus, NBCe1 function is crucial for ion and fluid transport in both the kidney and the eye. This patient with S427L also presents with abnormal dentition not found in other family members. She has normal intelligence rather than retardation associated with other NBCe1 mutations (11,26). Finally, P$_{\text{CO}_2}$ levels are abnormally high for the patient’s blood pH and [HCO$_3^-$] indicating a probable respiratory acidosis in addition to the pRTA rather than the respiratory system compensating for the pRTA.

S427L and the other NBCe1 mutations highlight that NBCe1 is the major HCO$_3^-$ absorption path for the PT and that NBCe1 is the dominant renal HCO$_3^-$ transporter. That is, the other acid-base transporters of the kidney, e.g., Cl$^-$-HCO$_3^-$ exchange and H$^+$ pump activities of the distal nephron can not compensate for loss or decreased NBCe1 function. We would expect better respiratory compensation, e.g., increased ventilation causing decreased blood P$_{\text{CO}_2}$, thereby increasing blood pH. Thus, poor or lacking compensation in S427L and other NBCe1-mutation patients, indicates that the NBCe1 defect may also impair other systemic acid-base homeostatic mechanisms. For example, NBCe1 is found in CNS glia and neurons (27-29). Perhaps NBCe1 plays a crucial role in feedback control mediated by the chemoreceptor neurons of the carotid body, glomus cells and/or the brain respiratory center or CO$_2$/HCO$_3^-$ exchange by the lung epithelia.

Ocular defects with NBCe1 mutations (S427L, R298S, R510H) indicate that NBCe1 is critical for fluid homeostasis (glaucoma) and transparency (cataracts) in the eye. Two groups have localized eye NBCe1 isoforms (30,31). Bok and coworkers found that NBCe1-B (pNBCe1) expressed throughout the rat eye (ciliary body, conjunctiva surface cells, cornea, lens epithelium, and retina), while NBCe1-A (kNBCe1) was only present in the conjunctiva basal cells. These investigators postulated that all the eye pathologies are associated with mutations in pNBCe1.
Usui et al. used different NBCe1 isoform antibodies on human eyes. They found that k- and p-NBCe1 are both present in human eye. Recently a non-sense mutation (Q29X) which removes only kNBCe1 was discovered in a patient with pRTA and glaucoma but no cataract or keratopathy (10). This patient’s phenotype reveals that kNBCe1 loss-of-function is sufficient to cause both pRTA and glaucoma, implying other eye pathologies are due to pNBCe1 loss. As defects of kNBCe1 increase intraocular pressure, its normal role in the eye must be to move NaHCO$_3$ out of the anterior chamber.

S427L resides in a domain common to k-/p-NBCe1, yet no clinical evidence of pancreatic dysfunction in our patient exists and amylase levels are always in the normal range. Other tissues which use NBCe1 to move Na$^+$ and HCO$_3^-$ into cells (e.g. intestine, heart) are also apparently spared. We speculated that NBCe1 function is not critical due to redundant transport mechanisms in these tissues. Fig 1a illustrates a dental phenotype associated with the S427L mutation. That is, amylase secretion may not be predictive of salivary or even pancreatic NaHCO$_3$ secretion. Several groups have reported NBCe1 protein in basolateral membrane of salivary glands (parotid and submandibular) (32-34). In salivary glands, the NBCe1-B but not NBCe1-A is found. In this secretory tissue as in the pancreas, NBCe1 mediates the uptake of NaHCO$_3$ from the blood into the cell. Our experiments illustrate that at -60 mV, NaHCO$_3$ influx mediated by S427L-NBCe1 is reduced by ~10-fold compared to wt. In the pancreas this activity, now attributed to NBCe1-B, accounts for 75% of HCO$_3^-$ uptake for secretion (35). Salivary glands seem to mirror these activities. Salivary HCO$_3^-$ secretion buffers acids (H$^+$) generated from oral bacterial fermentation. Thus, decreased salivary HCO$_3^-$ secretion, should accelerate tooth decay and compromise dentition. In Sjögren Syndrome (OMIM #270150), expression of Cl$^-$-HCO$_3^-$ exchange, the apical HCO$_3^-$ transport step, is lost. Among the Sjögren phenotypes are salivary gland dysfunction leading to poor dentition.

Poor dentition may also be NBCe1-phenotypic because oral carbonic anhydrase inhibition affects mineralization of tooth enamel (36). Recently, NBCe1 has been demonstrated to interact with CAII/CAIV (37,38). The physiologic role of this CA-NBCe1 interaction in the kidney is not.
understood. Inhibition of renal CA (acetazolamide) is known to reduce PT bicarbonate absorption and can lead to metabolic acidosis. Grichtchenko & Boron provided experimental evidence that CA-NBCe1 interaction reveals CO$_3^{2-}$ transport by NBCe1 (39). Given these data, CA inhibition and NBCe1 mutations should present with some similar phenotypes.

Overall, the S427L activity reduction is predicted to also decrease salivary HCO$_3^-$ secretion. Poor dentition is found in our S427L patient but not the family (Fig 1). Whether severe tooth decay is a S427L result specifically, NBCe1 mutations in general or prolonged, chronic acidosis is unclear. Dental phenotypes have not been reported for other NBCe1 mutations, however in 1979 Winsnes reported brothers with pRTA, bilateral glaucoma & cataracts, short stature, and poor dentition (40). Unfortunately, these individuals are not available for molecular analysis.

**The S427L transport defect**

We have evaluated S427L protein expression and transporter function using *Xenopus* oocyte expression. The S427L protein traffics normally to the plasma membrane similar to wt-hkNBCe1 (Fig 2) (17). Our pH$_i$ and voltage clamp experiments reveal that S427L has about 10% of wt-NBCe1 function (Fig 3, Fig 4). We predict that either kinetics or capacity (apparent affinity) of S427L is altered vs. wt. Wt-NBCe1 is a low affinity / high capacity transporter (21). NBCe1 affinity for Na$^+$ is 30 mM (21), and affinity for HCO$_3^-$ is ~6.5 mM (41). Reducing affinity is not predicted to reduce whole cell currents as measured here, unless there is an interdependence of Na$^+$ and HCO$_3^-$ “binding.” Previous experiments with rkNBCe1 indicated that apparent K$_m$ for Na$^+$ is voltage and [HCO$_3^-$] independent (21).

Our results indicate that S427L has both voltage and Na$^+$ dependent transport defects. Analysis of the S427L V$_m$ dependence of the HCO$_3^-$ stimulated current (I$_{HCO3}$) and the Na$^+$ dependent HCO$_3^-$ stimulated current (I$_{0NaHCO3}$) provides a more accurate description of S427L dysfunction. Thermodynamics allows us to calculate an E$_{rev}$ and predict in which direction NaHCO$_3$ should move by knowing ion concentrations and V$_m$ (21). However, the biophysical defect of S427L is such, that with Na$^+$, even -160mV does not elicit NaHCO$_3$ efflux (Fig 4, Fig
S427L–NBCe1 is an ion transport defect

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5). These data imply that either a voltage sensor or a Na⁺ binding site of S427L is defective. We speculate that the location of S427L, the beginning third of TM1

(…YDALNIQA(L_SA)ILFIYLATVTAITFGGLL…), contributes to the lack of a S427L-E_{\text{rev}}. A pile-up of the animal members of the S1c4 gene family (excluding BTR1, a.k.a. SLC4A11, since it is very different and transport is unknown) reveals that the “L(S)A” sequence among the S1c4 family is only modestly changed⁸: LAA, LAS, LSA (NBCe1), ISA, IAA, VAA, MAA, FAS, FSA, LST (fugu-clone, Romero unpublished). Interestingly, “LSA” is found in all NBCe1 clones, while “ISA” is found in all NBCe2 (NBC4 / S1c4A5) clones to-date, implying that lack of side-chain bulk (S or A) may interfere with TM-helix packing or ion permeation. In support of this conjecture are our preliminary data with a “SLA” double NBCe1 mutant (L426S-S427L-NBCe1, not shown) that is functionally dead. However, helical wheel predictions indicate that the TM1 hydrophobic character/packing is not altered by the S→L transition. That is, S427L is unlikely to cause a structural problem per se. S427T reveals roughly wild-type transport function (not shown), implying that –OH chemistry at S427 is important for normal function. Perhaps helix interaction, via the –OH side-chain or packing, controls voltage sensing or Na⁺ “unbinding / dissociation” at the NBCe1 intracellular face.

Our model may not encompass the extended complexity of the NBCe1 mutation phenotype. 0Na-HCO₃ reveals another aspect to the S427L defect. Wt-hkNBCe1 currents show strong rectification whether expressed as a difference to non-HCO₃⁻ solution (I_{0NaHCO₃} – I_{ND96}, Fig 4) or to NaHCO₃ solution (I_{0NaHCO₃} – I_{HCO₃}, Fig 5b). S427L data analysis reveals a small, voltage-dependent current associated with reversal of the Na⁺ chemical gradient. I_{0Na}^{S427L} does not rectify but a near linear voltage dependence and an E_{\text{rev}} (Fig 5b). However, I_{0NaHCO₃}^{S427L} is virtually voltage-independent (Fig 4). Nevertheless, experiment summaries in Figs 3-5 reveal a small outward current compared to the HCO₃⁻ state. Since I_{0Na}^{S427L} does not exceed background currents observed in the absence of CO₂, this current could represent true NaHCO₃ efflux, a

⁸ S1c4a1 (AE1): LAA; S1c4a2 (AE2): LAA, IAA, MAA; S1c4a3 (AE3): VAA; S1c4a4 (NBCe1): LSA; S1c4a5 (NBC4 / NBCe2): ISA; S1c4a7 (mNBC3, NBCn1): LAS (ceNBC is also LAS); S1c4a8 (NDCBE1, kNBC3): LAS; S1c4a9 (AE4, NBCn2): VSA, FSA; S1c4a10 (NCBE): LAS; Dros NDAE1: VAS
dissociation of charges from NBCe1 or merely decreased NaHCO₃ influx. However, our ΔpHᵢ data (Table 2) indicate that NaHCO₃ efflux of S427L is only ~0.3% (not 10% as for NaHCO₃ influx) of wt-hkNBCe1.

In summary, we have identified a novel missense mutation (S427L) in NBCe1 located at or near the beginning of TM1. This recessive mutation is phenotypically manifest in the kidney (pRTA), eye (cataracts, glaucoma, band keratopathy), teeth and perhaps the respiratory system. Interestingly, this patient has no mental retardation. Transport experiments reveal that S427L has ~10% wt-NBCe1 activity for NaHCO₃ influx. S427L operates poorly in the NaHCO₃ efflux mode necessary for renal HCO₃⁻ absorption and ocular fluid transport in anterior chamber. That is, S427L-NBCe1 is a severe ion transport defect.

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FIG LEGENDS

**Fig 1: S427L-NBCe1 mutation.**

a, patient’s facial manifestations (top, blind eye with cataract and corneal opacity; bottom, abnormal dentition). b, illustrates the sequence analysis of C→T transition (see Methods): b1, Normal; b2, mother; b3, patient. c, ethidium bromide stained 3% agarose gel showing the Taq/I digest of panel b. PCR amplification using the primers F8 and R9 yielded a 92 bp fragment (upper arrow). Following PCR, products were digested with Taq/I to yield 25 bp (not shown) and 67 bp (lower arrow) fragments in wt individuals. The C→T transition abolishes this restriction site. M, marker; A, normal; B, mother; C, patient, D, sister, E, normal.

d, Analysis of polymorphic markers on chromosome 4q21 was performed using fluorescent primers. Genomic distances are indicated in centi-Morgans (cM using deCODE map) and Mega base pairs(Mbp using the STS map numbering). Markers D4S398 (4.7 cM, 10.4 Mbp) and D4S392 (1.5 cM, 1.8 Mbp) are 5’ to the sense direction of SLC4A4. Markers D4S2964 (4.7 cM, 8.3 Mbp) and D4S1534 (6.6 cM, 13.8 Mbp) are 3’ of SLC4A4. For each family member, two alleles in the SLC4A4 region were created. The father’s alleles are predicted. Disease carrying alleles are bolded. The patient is homozygous for D4S392 (1.5 cM from SLC4A4), but heterozygous for other markers in the area. A recombination is seen in the sister between D4S392 and D4S2964 (underlined). The patient and her sister inherited different alleles from the father.

**Fig 2: NBCe1 expression**

First, oocytes were used for electrophysiology experiments (pH, and unclamped V.). After electrophysiology, the oocyte was embedded in OCT, cryosectioned, and stained with a NBCe1 antibody and visualized by epifluorescence (bottom). Representative results are shown for controls (Water-injected), hkNBCe1 and S427L. The left panel illustrates that water-injected oocytes have no NB Ce1 activity and no NB Ce1 protein. The middle panel shows that hkNBCe1
Oocytes function as electrogenic Na\(^+\)/HCO\(_3\)\(^-\) cotransporters and express the NBCe1 protein at the plasma membrane. The right panel illustrates that S427L oocytes have lower NBCe1 activity but immunoreactivity is similar. Lower case letters mark solution changes ("a" to "e" for pH\(_i\) trace; "a'" to "e'" for V\(_m\)) and are included designate specific areas of the individual experiments to make text descriptions more explicit. "030123pf," "030123pb" and "030123pe" designate the specific cell’s data illustrated.

**Fig 3: NBCe1 expression (pH\(_i\) and voltage clamping)**

Oocytes were treated as indicated in Fig 2 and another KCl electrode was added to clamp at -60 mV while measuring pH\(_i\). Solution changes are indicated by bars and "a-e" on each panel: left is a water injected control, middle is hkNBCe1, and right is S427L. Lower case letters have the same meaning as in Fig 2 except "a'" to "e'" indicate current. Capital letters ("A" to "D") refer to time points for which current-voltage (I-V) responses were analyzed (see Fig 4). "031007fd," "031007fa" and "031006fe," designate the specific cell’s data illustrated.

**Fig 4: NBCe1 I-V relationships**

Oocytes were clamped at -60 mV. Voltage step protocols were executed 30-60 seconds before a solution change. Step protocols were run at peak currents (A in Fig 3; diamonds) and at 5 min (B in Fig 3; squares). I\(_{\text{HCO}}\) was calculated as I(HCO\(_3\)\(^-\)) – I(ND96) (21). Likewise, the I\(_{\text{NaHCO}}\) was calculated from I(0Na-HCO\(_3\)\(^-\), “C” in Fig 3) – I(ND96) as previously (21) and shown in triangles (dashed line). Finally, the “X” curve was calculated from I(ND96 at end, “D” in Fig 3) – I(ND96, start) representing recovery after HCO\(_3\)\(^-\) washout. Average responses are indicated: water (n=10); hkNBCe1 (n=9); S427L (n=25).

**Fig 5: Comparison of hkNBCe1 and S427L-hkNBCe1 HCO\(_3\)\(^-\) dependent current with and without Na\(^+\)**

The data set is the same as Fig 4. a illustrates I\(_{\text{NaHCO}}\) data (“B,” Fig 3 @ stable pH\(_i\)). Wt data is indicated by closed squares (solid line). S427L data is indicated by “X” (dashed line) and is
plotted on a 0.1-fold of wild-type scale (nA-scale). b is a plot of $I_{0\text{Na}}$, $I(0\text{Na-HCO}_3^-$, C in Fig 3) – $I(\text{HCO}_3^-$, B in Fig 3) as a different view of the Na$^+$ dependent HCO$_3^-$ current.
Table 1: Non-voltage clamped transport measurements

| units       | Water (mV) |     |     |     | hkNBCe1 (mV) |     |     |     | S427L (mV) |     |     |
|-------------|------------|-----|-----|-----|--------------|-----|-----|-----|------------|-----|-----|-----|
| mV          | initial $V_m$ | -40.4 | 5.4 | 11  | -39.0 | 2.9 | 26  | -48.8 | 2.4 | 25  |
|             | Initial $pH_i$ | 7.28  | 0.08 | 8   | 7.46  | 0.06 | 14  | 7.32  | 0.06 | 13  |
|             | Final $pH_i$  | 6.96  | 0.02 | 4   | 7.11  | 0.08 | 8   | 6.94  | 0.04 | 3   |
|             | $\Delta pH_i$ | -0.45 | 0.01 | 4   | -0.44 | 0.03 | 8   | -0.49 | 0.02 | 3   |
| mM          | $[HCO_3^-]_i$ | 8.0   | 0.3  | 4   | 13.9  | 1.9  | 6   | 7.6   | 0.6  | 3   |
| mM/pH unit | Apparent $\beta_T$ | 18.0  | 1.0  | 4   | 32.1  | 5.6  | 6   | 15.7  | 1.9  | 3   |
| mM/pH unit | Apparent $\beta_{CO_2}$ | 18.5  | 0.7  | 4   | 32.1  | 4.5  | 6   | 17.5  | 1.5  | 3   |
| dp$pH_i$/dt | (x10^{-5} pH unit/s) | 127.0 | 21.5 | 4   | 148.4 | 12.3 | 8   | 130.7 | 8.8  | 3   |
| dp$pH_i$/dt | $CO_2$ (dp$pH_i$/dt) | -277.0 | 37.9 | 4   | -305.7 | 27.6 | 8   | -240.0 | 18.7 | 3   |
| dp$pH_i$/dt | 0-Na$^+$-CO$_2$ | 1.8   | 6.8  | 4   | -45.3 | 14.8 | 6   | -34.3 | 47.2 | 3   |
| dp$pH_i$/dt | ND96       | 127.0 | 21.5 | 4   | 148.4 | 12.3 | 8   | 130.7 | 8.8  | 3   |

Calculations are as indicated in Experimental Procedures and Results. $V_m$ is membrane potential. For these data, oocytes are not voltage clamped. $\Delta \beta_{hkNBCe1}$ is $\beta_T(hkNBCe1) - \beta_T(water)$. 
Table 2: Membrane current ($I_m$) & Intracellular activity for oocytes clamped at -60mV

<table>
<thead>
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<th>units</th>
<th>Water</th>
<th>hkNBCe1</th>
<th>S427L</th>
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<tr>
<td></td>
<td>avg</td>
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<td>dpH/dt (x10$^{-5}$ pH unit/s)</td>
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<td>CO$_2$/HCO$_3^-$</td>
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<td>0Na$^+$-CO$_2$</td>
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<td>6</td>
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<td>26.7</td>
<td>6</td>
</tr>
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</table>

These data are similar to Table 1 except that this distinct dataset was collected using the 3-electrodes experiments (see Experimental Procedures) to voltage clamp oocytes at -60 mV while also measuring pH$_i$. $I_m$ is membrane current. For pH$_i$’s and ΔpH$_i$’s, there are actually four significant figures though three are shown for readability.
Figure 1 – Dinour, Chang et al.

**a**

![Image of a person's eye and teeth]

**c**

![Image of genetic analysis bands]

**d**

![Genetic analysis table]

<table>
<thead>
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<th>Father</th>
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<td>2 3</td>
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<td>3 1</td>
<td>1 2</td>
<td>1 2</td>
</tr>
</tbody>
</table>

`b1`, `b2`, `b3` represent different genetic analysis plots.
Figure 3 – Dinour, Chang et al.

Water

hkNB Ce1

S427L

CO₂/HCO₃⁻

0Na⁺

pHᵢ

nAmp

5 min

031007fd

5 min

031007fa

5 min

031008fe
Figure 4 – Dinour, Chang et al.

Water

hkNBCe1

S427L

-300 -200 -100 0 100 200 300

mV

-200 -150 -100 -50 0 50 100

mV

-300 -200 -100 0 100 200 300

mV

-200 -150 -100 -50 0 50 100

-300 -200 -100 0 100

nA

33 HCO$_3$-A

33 HCO$_3$-B

0Na-HCO$_3$

ND96-w
Figure 5 – Dinour, Chang et al.

(a) hkNBCe1
- - S427L

(b) hkNBCe1
- - S427L
A novel missense mutation in the sodium bicarbonate cotransporter (NBCe1/SLC4A4) causes proximal tubular acidosis and glaucoma through ion transport defects
Dganit Dinour, Min-Hwang Chang, Jun-ichi Satoh, Brenda L. Smith, Nathan Angle, Aaron Knecht, Irina Serban, Eli J. Holtzman and Michael F. Romero

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